Copper Deficiency Decreases Complex IV but Not Complex I, II, III, or V in the Mitochondrial Respiratory Chain in Rat Heart[1]

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Abstract

It has been documented that dietary copper (Cu) deficiency impairs mitochondrial respiratory function, which is catalyzed by 5 membrane-bound multiple protein complexes. However, there are few reports on the simultaneous analysis of Cu effect on the subunit protein expression on all 5 protein complexes. The present study was undertaken to determine the effect of Cu deficiency on each mitochondrial respiratory complex's protein expression in rat heart tissue with western-blot analysis. Male Sprague-Dawley rats were fed diets that were either Cu adequate (6.0 μg Cu/g diet, $n = 5$) or Cu deficient (0.3 μg Cu/g diet, $n = 5$) for 5 wk. The monoclonal antibody-based western-blot analysis suggested that the protein levels of 39-kDa and 30-kDa subunits in complex I; 70-kDa and 30-kDa subunits in complex II; core I and core II subunits in complex III; and α and β subunits of F1 complex in complex V in both high-salt buffer (HSB) and low-salt buffer (LSB) protein fractions from heart tissue of Cu-deficient rats did not differ from those of Cu-adequate rats. However, the protein level of cytochrome c oxidase (CCO) subunit (COX) I, COX Vb, and COX VIb subunits in complex IV (CCO) in both HSB and LSB protein fractions from heart tissue of Cu-deficient rats was lower than those of Cu-adequate rats. Collectively, these data demonstrate that Cu deficiency decreases each tested subunit protein expression of complex IV but not those of complex I, II, III, and V in mitochondrial respiratory complexes.   J. Nutr. 137: 14–18, 2007.

Introduction

Impaired mitochondrial function and energy production in copper (Cu)-deficient hearts are implied by a number of reports showing swelling and ultrastructural changes in cardiac mitochondria (1–4) and depression of cytochrome c oxidase (CCO)[2] activity (5–7). Although ultrastructural changes and reduction of CCO activity suggest that mitochondrial energy production is impaired, early studies of cardiac mitochondrial function during Cu deficiency showed no abnormalities in ADP:O ratios, ATP concentrations, or respiration (8–10). However, more recent studies have shown that Cu deficiency produces a slight depression of mitochondrial ATP and phosphocreatine concentrations, depressed respiration, and less efficient oxygen utilization (11–16). These studies indicate that Cu deficiency produces abnormalities in the electron transport chain and oxidative phosphorylation in cardiac mitochondria.

Mitochondrial ATP synthesis is dependent on the transfer of electrons between 4 oligomeric enzymes that comprise the respiratory complexes of the mitochondrial electron transport chain. These 4 enzymes are: NADH:ubiquinone oxidoreductase (complex I), succinate:ubiquinone oxidoreductase (complex II), ubiquinol:cytochrome c oxidoreductase (complex III), and ferrocytochrome c:oxygen oxidoreductase (complex IV, CCO). Electrochemical energy derived from electron transfer between the respiratory complexes and finally to molecular oxygen drives the translocation of protons across the inner mitochondrial membrane, which creates a transmembrane protein gradient and membrane potential. The proton-motive force provided by the gradient and membrane potential is utilized by F_1F_0 ATPase (complex V) to synthesize ATP (17).

CCO is a cuproenzyme that serves as the terminal respiratory complex (complex IV) of the mitochondrial electron transport chain. The reduction of CCO activity in hearts of Cu-deficient animals (5–7) is not an unexpected outcome because Cu is an essential cofactor. However, additional mechanisms may also contribute to the loss of cardiac CCO activity. It has been reported that Cu deficiency reduces the content of CCO subunits IV, V, and VIb (18–20), CCO protein, and heme associated with cytochrome aa3 (21) in cardiac mitochondria. CCO deficiency has also been reported in the hearts of Cu-deficient rat neonates (22). Thus, defective CCO assembly or impaired holoenzyme stability also may contribute to the decline in CCO activity caused by Cu deficiency.

Several reports indicate that Cu deficiency decreases the activities of NADH cytochrome c reductase, representing combined activities of complexes I and III, succinate cytochrome c reductase, representing the combined activities of complexes II and III (23,24) and alters the subunit content of F_1F_0 ATPase (1,18,25). These findings indicate that in addition to its effect on CCO, Cu deficiency may produce a more global effect on respiratory complexes involved in electron transport and oxidative phosphorylation. Recent work with yeast, beef, and plants has provided evidence that mitochondrial electron-transfer complexes specifically interact to form supermolecular structures called supercomplexes (26–28). Experimental findings have shown that respiratory supercomplexes allow higher electron-transfer rates (28,29). Therefore, the protein ratio of respiratory chain complexes I:II:III:IV:V is critical for the assembly of respiratory supercomplexes and optimization of mitochondrial electron transport and oxidative phosphorylation. It is possible that reduction in CCO protein or subunit content contributes to the reported adverse effects of Cu deficiency on cardiac electron transport, energy production, and complex activities by impairing supercomplex formation. However, none of the previous studies have made a systematic comparison of all 5 mitochondrial respiratory complexes I:II:III:IV:V in a single animal study, which is the groundwork for studying the assembly of respiratory supercomplexes. Recent availability of monoclonal antibodies (mAbs) against the subunit proteins of mitochondrial respiratory complexes facilitates the study of subunit level expression during Cu deficiency (30). This study examined the protein expression level of at least 2 key subunits for each respiratory complex and the data provide new insights into the regulation of protein expression related to mitochondrial respiratory function of the Cu-deficient rat heart.

Materials and Methods

Animals and diets. Experiments were conducted in accordance with the Guide for the Care and Use of Laboratory Animals (31) and approved by the Animal Care Committee of the Grand Forks Human Nutrition Research Center.

Twenty male, 3-wk old, weanling Sprague-Dawley rats (Charles River/Sasco) were divided into 2 dietary groups. Diets were composed of 940 g Cu-free, iron (Fe)-free basal diet (catalogue no. TD 84469, Teklad Test Diets); 50 g safflower oil; and 10 g Cu-Fe mineral mix per kg of diet. The basal diet was a casein- (200 g/kg), sucrose- (386 g/kg), cornstarch- (295 g/kg) based diet containing all known essential vitamins and minerals except Cu and Fe (32). The mineral mix contained cornstarch and Fe with or without Cu and provided 0.22 g ferric citrate (16% Fe) and either 0 or 24 mg added $CuSO_4·5H_2O$/kg of diet. These formulations were intended to provide a severely Cu-deficient diet (CuD) containing only Cu present in the basal diet and a Cu-adequate diet (CuA) containing 6 mg/kg diet. Triplicate dietary analyses (see below) of each diet indicated mean Cu concentrations of 0.16 and 6.26 mg Cu/kg diet for the CuD and CuA diets, respectively.

Analysis of dietary Cu was performed by dry ashing of the diet sample (33), dissolution in aqua regia, and measurement by atomic absorption spectroscopy (model 503, Perkin Elmer). The assay method was validated by simultaneous assays of a wheat flour reference standard (National Institute of Standards and Technology) and a dietary reference standard (HNRC-1A) that was developed by the Grand Forks Human Nutrition Research Center.

After the rats consumed their respective diets for 5 wk, each rat was anesthetized with an intraperitoneal injection of sodium thiobutabarbital (Inactin, Research Biochemicals International; 100 mg/kg body wt). Blood was withdrawn from the inferior vena cava into EDTA-treated test tubes and hemoglobin and hematocrit were determined with a cell counter (Cell-Dyn, Model 3500CS, Abbott Diagnostics). The median

lobes of the liver (10 rats from each dietary group) and hearts (5 rats from each dietary group) were excised for mineral assays. Liver/heart Cu concentrations were determined by lyophilizing and digesting organ samples with nitric acid and hydrogen peroxide (34) and measuring Cu concentration by inductively coupled argon plasma emission spectroscopy (Model 1140, Jarrell-Ash).

Hearts from 5 rats from each dietary group were excised and placed in PBS on ice for subsequent protein extraction, described below.

Preparation of low-salt buffer and high-salt buffer protein extracts. Unless otherwise indicated, all operations were performed at 4°C. Low-salt buffer (LSB) and high-salt buffer (HSB) protein extracts were prepared by a generally accepted procedure (35) as modified for use in a previous study (20). Fresh tissues from heart muscle were finely minced in PBS and centrifuged at $532 \times g$; 5 min. The pellets were lysed in lysis buffer [20 mmol/L HEPES, pH 7.6, 20% glycerol, 10 mmol/L NaCl, 1.5 mmol/L $MgCl_2$, 0.2 mmol/L EDTA, 1 mmol/L dithiothreitol, 1 mmol/L phenylmethylsulfonyl fluoride, and leupeptin (10 mg/L)] in a Wheaton Dounce homogenizer. Nuclei and other organelles were collected by centrifugation at $532 \times g$; 5 min and supernatant was designated the LSB protein extract and kept at −80°C. Nuclei and organelles were suspended in lysis buffer containing 500 mmol/L NaCl, gently rocked for 1 h, and then centrifuged at $15,000 \times g$; 15 min. The supernatant was designated the HSB protein extract and kept at −80°C. The LSB and HSB extracts represent the total cellular protein, and data from parallel analysis of both LSB and HSB extracts may be more informative than that of a single whole cell extract.

Western-blotting analysis. Equal amounts of HSB or LSB protein extract (2.5 µg/lane) were resolved over 4–20% Tris-glycine gradient gels under denaturing and reducing conditions and electroblotted onto polyvinylidene difluoride membranes (Invitrogen). The identical SDS gels (after transferring protein to polyvinylidene difluoride membrane) were stained with Coomassie Blue to ensure equal loading, because there was always a certain percentage of protein still remaining in these gels (20,36). Membrane blots were blocked in PBS – 0.05% Tween (v:v) supplemented with 1% (wt:v) nonfat dry milk (BioRad) at room temperature (RT) for 1 h. Membranes were probed with antibodies against 39-kDa and 30-kDa subunits in complex I; antibodies against 70-kDa and 30-kDa subunits in complex II; antibodies against core I and core II subunits in complex III; antibodies against CCO subunit (COX) I, COX Vb, and COX VIb subunits of complex IV; antibodies against α and β subunits of F1 complex in complex V (Molecular Probes) for 1 h at RT according the manufacturer's suggested concentration. Membranes were washed (2×1 min, 1×15 min, and 2×5 min) and then incubated with an anti-mouse (1:3000 dilution) horseradish peroxidase–conjugated secondary antibody (Santa Cruz Biotechnology) in blocking solution for 1 h at RT. Blots were washed as above and proteins were detected using an ECL plus kit (Amersham Pharmacia Biotech) with the Molecular Dynamics Image-Quant system.

Statistical analysis. Results are given as means ± SD. Student's *t* test for unequal variances was used to compare data between the 2 dietary treatments. Differences with a *P*-value < 0.05 were considered significant.

Results

Body weight, hematocrit, hemoglobin, liver Cu, and heart Cu were lower in CuD than in CuA rats (**Table 1**). In contrast, the ratio of heart weight vs. body weight was higher in CuD than in CuA rats. These data are characteristic of Cu-deficient rats, as we have previously reported (20,36–38). To determine the effect of Cu deficiency on the protein expression of mitochondrial complexes I, II, III, IV, and V, we systematically examined all 5 complexes with western-blotting analysis. Cu deficiency produced no changes on the protein expression of 39 kDa and 30 kDa in complex I (**Fig. 1, Table 2**); 70 kDa and 30 kDa in

TABLE 1 General features of Cu-adequate and Cu-deficient rats[1]

Diet	Body wt, g	Heart/Body,[2] g/kg	Hemoglobin, g/L	Hematocrit	Heart Cu,[2] nmol/g dry tissue	Liver Cu, nmol/g dry tissue
CuD	262.6 ± 27.9*	5.30 ± 1.07*	70.42 ± 16.0**	0.225 ± 0.048**	89.6 ± 17.0**	13.1 ± 3.9**
CuA	299.3 ± 21.1	3.49 ± 0.29	142.8 ± 4.08	0.437 ± 0.015	310.3 ± 11.2	176.4 ± 11.3

[1] Values are means ± SD, $n = 10$. *Different from CuA, *$P < 0.02$, **$P < 0.0001$, $n = 5$.
[2] $n = 5$.

complex II (Fig.1, Table 2); core I and core II subunits in complex III (Fig. 1, Table 2); or α and β subunits in complex V (Fig. 1, Table 2). However, Cu deficiency decreased the protein expression of COX I, COX Vb, and COX VIb subunits in complex IV by 44.2, 40.2, and 61.8%, respectively, in the HSB fraction and by 59.1, 51.9, and 35.8%, respectively, in the LSB fraction (Fig. 1, Table 2).
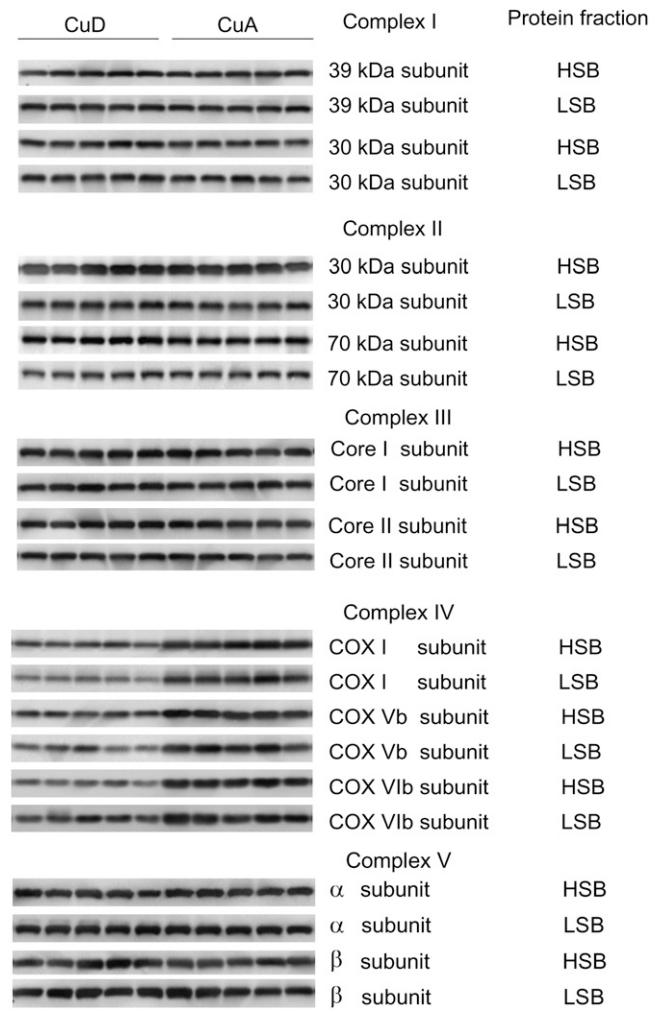
Discussion

To our knowledge, this study is the first to determine the effect of Cu deficiency on subunit content of all 5 respiratory complexes

Figure 1 Effect of Cu deficiency on the protein expression mitochondrial respiratory complexes. Western-blotting analysis of HSB and LSB heart-protein extracts from rats fed either a CuD or a CuA for 5 wk (5 rats per group). Each complex protein profile was done on individual membranes, and each lane was loaded with 2.5 μg of protein and individual subunits were detected by probing the blot with subunit-specific antibodies. The molecular weights of the immunoreactive bands were estimated from regression analysis using prestained molecular markers (Invitrogen).

in cardiac mitochondria in a single experiment. Our study showed that of the 5 cardiac mitochondrial respiratory complexes examined, Cu deficiency significantly lowered only the subunits of CCO (complex IV). This finding agrees with a previous observation that CCO protein in heart mitochondria is reduced by Cu deficiency, whereas protein content of complexes I, III, and V are unaffected (21). The affected subunits in our study included mitochondrial-encoded COX I and nuclear-encoded COX Vb and COX VIb. This finding is somewhat in contrast to previous research showing that Cu deficiency primarily lowered nuclear-encoded subunits of CCO (1). However, the finding is consistent with a recent study showing that levels of mitochondrial- and nuclear-encoded CCO subunits are reduced in cardiac mitochondria of Cu-deficient rat neonates (22) and with a recent study showing that Cu deficiency decreases the protein level of subunit VIb of CCO but not the protein level of subunit IP of complex II (20). It cannot be concluded that Cu deficiency specifically affects only CCO subunits, because the effect of Cu deficiency was examined in relatively few of the large number of subunits composing each of the 5 respiratory complexes (39). However, because mitochondrial- and nuclear-encoded subunits of CCO were both lowered, it may be concluded that a deficiency in complex IV content occurs in the hearts of Cu-deficient rats.

Mechanisms for CCO deficiency caused by Cu deficiency are not clear. However, an early study with Cu-deficient yeast indicated that Cu is important for assembly of CCO (40). Further support for impaired CCO assembly during Cu deficiency is provided by studies showing that cytochrome aa3 content is diminished in Cu-deficient rats (21,41). In yeast, the synthesis of heme a is not affected by intracellular Cu levels (42), suggesting that the reduction in cytochrome aa3 observed in Cu-deficient rats is a consequence of improper trafficking and incorporation of hemes a and a3 during CCO assembly. Our data suggest that the diminished content of COX I in Cu-deficient rats contributes to the reduction of cytochrome aa3, because heme a and a3 are located in the COX I subunit of CCO (43). Thus, our findings, together with the earlier data and data from a study showing that Cu deficiency reduces the content of CCO protein in the heart (21), indicate that Cu deficiency impairs the assembly of fully functional CCO in heart mitochondria.

CCO assembly has several sequential stages for the insertion of prosthetic groups and subunit associations. Cu delivery to the apoform of COX I occurs during the first stage of CCO assembly (44,45) and limited Cu availability due to Cu deficiency may impair this stage of assembly. Incomplete assembly of COX I can be detrimental to the complete assembly of the holoenzyme. Mitochondria have evolutionarily conserved metalloproteinases that remove nonassembled polypeptides and prevent their accumulation in the inner membrane (46). Thus, incompletely formed COX I in Cu-deficient animals may be degraded, causing an overall reduction in COX I content in the mitochondria. Also, in yeast, accumulation of unassembled COX I halts the synthesis of COX I through feedback inhibition of COX I gene translation

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TABLE 2 Cu deficiency and subunit expression levels of mitochondrial respiratory complexes

Complex	Subunit	Alternative name	Cellular fraction	Intensity units	
				CuD	CuA
I	39 kDa	α subcomplex, 9	HSB	375.8 ± 71.3	376.5 ± 27.8
			LSB	372.6 ± 21.3	357.8 ± 16.9
I	30 kDa	Iron-sulfur protein, 3	HSB	414.2 ± 57.3	369.1 ± 26.9
			LSB	376.1 ± 36.6	342.1 ± 51.8
II	30 kDa	Iron-sulfur protein	HSB	373.3 ± 68.1	370.9 ± 40.7
			LSB	415.4 ± 41.0	390.3 ± 29.5
II	70 kDa	Flavoprotein	HSB	374.5 ± 23.0	365.9 ± 35.8
			LSB	382.2 ± 48.5	405.9 ± 11.5
III	Core I	None	HSB	380.5 ± 38.7	365.2 ± 54.2
			LSB	376.5 ± 48.8	358.6 ± 26.5
III	Core II	None	HSB	366.7 ± 65.4	349.7 ± 46.1
			LSB	392.4 ± 32.3	375.8 ± 55.1
IV	I	COX I	HSB	366.1 ± 43.3*	656.7 ± 71.2
			LSB	186.7 ± 27.3*	456.0 ± 60.9
IV	Vb	COX Vb	HSB	337.0 ± 20.3*	563.5 ± 57.0
			LSB	187.6 ± 56.4*	390.0 ± 65.6
IV	VIb	COX VIb	HSB	370.0 ± 57.9*	968.8 ± 85.9
			LSB	371.7 ± 47.0*	579.2 ± 70.0
V	α	F1 complex, α subunit	HSB	315.8 ± 39.8	327.0 ± 31.3
			LSB	368.4 ± 35.8	378.7 ± 22.6
V	β	F1 complex, β subunit	HSB	367.9 ± 68.6	332.4 ± 22.8
			LSB	376.9 ± 33.8	371.8 ± 46.9

[1] Values are means ± SD, $n = 5$. *Different from CuA, $P < 0.001$.

(47). If a similar feedback mechanism exists in mammals, then reduced COX I content in Cu-deficient rats may occur through a combination of degradation and lower COX I protein synthesis because of lower incorporation of Cu into COX I during the early stage of CCO assembly. The reduction in nuclear encoded subunits in Cu-deficient rats may then occur, because less COX I is available for interaction with the nuclear encoded subunits during a later stage of CCO assembly.

The deficiency of CCO in cardiac mitochondria caused by poor Cu status may impair structural-functional associations between respiratory complexes that optimize electron transport. Since the first isolation of respiratory-chain complexes over 40 y ago (39), the concepts of how they are arranged within the membrane have evolved. In particular, several of the respiratory complexes specifically interact to form supermolecular structures termed supercomplexes (26,27). In mitochondria from beef heart, interactions between complexes I, III, and IV lead to the formation of supercomplexes, termed respirosomes (28). The supercomplexes detected in bovine heart mitochondria contain a complex I monomer, a complex III dimer, and a variable copy number of complex IV. The major supercomplex representing >50% of the total complex I in heart mitochondria has only 1 copy of complex IV, i.e. $I_1III_2IV_1$ (27). Several roles have been proposed for respiratory supercomplexes. These include substrate channeling, catalytic enhancement, sequestration of reactive intermediates (28), stabilization of protein complexes (48), increasing the capacity of the inner mitochondrial membrane for protein insertion (27,28), and generating mitochondrial cristae morphology (49). Thus, impairment of supercomplex assembly or altered stoichiometric relations between complexes I, III, and IV in the supercomplexes may influence mitochondrial electron transport and morphology.

The importance of complex IV to the activities of complexes I and III following their assembly into supercomplexes has recently been demonstrated (50). The activity of complex I in supercomplex I_1III_2 was ~40% of that in supercomplex $I_1III_2IV_1$. Complex III activity in supercomplex I_1III_2 was only ~6% of that in supercomplex $I_1III_2IV_1$. Although supercomplex assembly and stoichiometry were not investigated in this study, it is conceivable that complex IV deficiency caused by Cu deficiency could limit the assembly of supercomplex $I_1III_2IV_1$ and prevent complexes I and III from attaining their optimal activities. Limitation in the assembly of supercomplex $I_1III_2IV_1$ during Cu deficiency is consistent with the loss of complex I-III activity previously reported in Cu-deficient HL-60 cells (23) and hepatic mitochondria of Cu-deficient rats (24). However, reasons for complex IV deficiency during Cu deficiency and the impact of complex IV deficiency on supercomplex formation remain to be elucidated.

In summary, our results demonstrate that Cu deficiency decreases each tested subunit protein expression (mitochondrial- and nuclear-encoded subunits) of complex IV but not those of complex I, II, III, and V in mitochondrial respiratory complexes and lay the groundwork for studying Cu regulated-assembly of respiratory supercomplexes.

Acknowledgments

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